Reduction of lesion growth rate of late blight plant disease in transgenic potato expressing harpin protein*

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Received May 15, 1998

Abstract Using harpin protein gene from apple fire blight pathogen *Erwinia amylovora* and potato *prp*1-1 promoter as main DNA elements, the feasibility of using pathogen infection-induced hypersensitive response was explored as a new strategy of engineering fungal disease resistance. Three plant transformation vectors were constructed and 68 transgenic potato plants were produced through *Agrobacterium* mediated transformation method. Southern, Northern and Western blot analysis demonstrated the insertion, transcription and protein expression of harpin protein gene in transgenic plants. Disease resistance test using a complex race of *Phytophthora infestans* as challenging pathogen showed that both constitutive and pathogen infection-induced expression of harpin protein gene in transgenic potato reduced the lesion growth rate of fungus. Among plants where harpin protein gene expression was induced only by fungus infection, two plants were found to be highly resistant to *P. infestans* infection. Fungal hyphae were not produced on total of 30 inoculated leaves from the two resistant plants and necrotic lesion was limited to inoculation area. The results highlighted that engineered hypersensitive response in plants was a very promising approach to produce fungal disease resistant genotype.

Keywords: fungal disease, hypersensitive response, harpin, potato late blight.

The hypersensitive response produced by plants to pathogen infection was one of active defensive reactions and was thought as the most powerful defensive way found in nature. It was an attempt to explore new approaches of engineering hypersensitive response to pathogen infection[1]. Based on the "gene for gene" relationship explaining interactions between plants and pathogens, de Wit proposed a new approach of controlling plant disease focusing on the engineered hypersensitive response, called two components system of non-specific resistance[2]. Recent experimental evidence indicated that recognition in the same cell of products of plant resistance gene (R) and corresponding pathogen avirulence gene (avr) resulted in cell hypersensitive necrosis[3,4], however, pathogen infection-induced cell death can not only keep whole plant healthy, but also limit the development of pathogen in inoculated leaves[5].

Harpin, a membrane associated protein, was encoded by *hrpN*, a member of *hrp* (hypersensitive response and pathogenecity) gene family in plant pathogen bacterium[6] and isolated from apple fire blight bacterium *Erwinia amylovora*. Purified harpin protein, when being penetrated into intercellular space of leaves of solananae plants such as tobacco and tomato, induced cell hypersensitive necrosis around the penetrating area[6]. Therefore, it is worthwhile to demonstrate if harpin protein gene driven by pathogen infection induced promoter could be used to form an effective disease resistant system in transgenic plant. Targeting on potato late blight caused by *P. infestans*, the authors provide evidence to indicate that engineered hypersensitive response is a very useful approach to develop disease resistant genotype.

* Project partly supported by the Molecular Biology Laboratory of CPRO-DLO in Netherlands.

1 Materials and methods

1.1 Materials

The plant materials used in our experiments included potato cultivar "Desiree" provided by Dr. Qu Dongyu in Vegetables and Flowers Institute of Chinese Academy of Agricultural Sciences. Genomic DNA of *Nicotiana tabacum* cultivar "Xanthi-nc" was provided by Dr. Pang Shengzhi in Cornell University. Plasmid pRLGEMB2 containing harpin protein gene coding region was constructed by the authors[7]. pBI525 containing gene expression cassette composed of double CaMV 35S promoter, Ω sequence from AMV, and NOS terminator was purchased from PBI Company of Canada. T-DNA vector pBINPLUS[8], potato diploid line LineV and R2, and *P. infestans* isolate 90128 (race 1, 3, 4, 6, 7, 8, 10, 11) were provided by Dr. Andy Pereira and Dr. Vivianne Vleeshouwers in CPRO-DLO, the Netherlands. Other plasmids were maintained by our laboratory.

1.2 Methods

1.2.1 PCR and molecular cloning. Methods for PCR and molecular cloning referred to that described by Sambrook et al.[9].

1.2.2 Potato transformation. Methods for PCR and molecular cloning referred to that described by Visser et al.[10].

1.2.3 Molecular detection of transgenic plants. The methods for extraction of DNA, RNA, Southern and Northern hybridization followed that described by Sambrook et al. with some modification[9]. For detection of protein expression using dot blot-ELISA, 100 mg leave tissue was collected and ground in 100 μL 1×PBS buffer containing 140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4, pH 7.3. The supernatant of centrifuged samples was diluted 10 times for use. The rabbit antibody against harpin protein was produced by the authors. The second antibody was goat anti-rabbit antibody labeled by alkaline phosphatase purchased from Sigma Company. NBT/BCIP system was used to indicate the reaction. For Western blot analysis, total protein, intercellular protein and intracellular protein were isolated respectively according to the methods described by de Wit and Spikman[11]. SDS-PAGE and Western blot hybridization followed the methods described by Sambrook et al.[9].

1.2.4 Disease resistance test. Totally 55 morphologically normal and healthy transgenic plants were used for disease resistance test. Both plants regenerated directly from stem and transformants containing empty vector pBINPLUS were used as negative control. Late blight susceptible cultivar "Bintje" was used to test the pathogenecity of *P. infestans* isolate.

The methods for preparation of culture medium for *P. infestans* growth and induction of zoospores referred to that described by Caten and Jinks[12]. The concentration of zoospores was 50 000 per mL. Biological assay was done using detached leaves from plants of 8-week old in a greenhouse. From top of each plant the third, fourth, and fifth of fully expanded leaves were collected. There are 5 compound leaves in each potato leaf. Only one site for each compound leaf on back surface was inoculated. There are a total of 15 inoculation sites for each transgenic plant with 10 μL of inoculation (total of 500 zoospores). The temperature in culture room for infection and growth of inoculated *P. infestans* was 18℃ at light for 16 h and 15℃ at dark for 8 h. Relative

humidity in culture room was 100%.

The disease lesion size was measured on the fourth, fifth and sixth days after inoculation for 3 times. The lesion area (A) was calculated based on the following formula: $A = 1/4 * \pi * \text{length} * \text{width}$. If the lesion size was limited in the inoculation site, i.e. $0 < A < 16\ mm^2$, the infection was not successful. If lesion area across 3 times of measurements was below that of inoculation area, the hypersensitive response happened; if lesion area was over 16 mm^2 at one time of 3 measurements, the infection was successful. For successful infection, the average of lesion radius calculated from lesion area for 3 measurements was converted into lesion growth rate (LGR) indicated as mm/day. The statistical analysis of LGR was done using ANOVA.

2 Result

2.1 Construction of harpin protein gene expression vectors and potato transformation

Harpin gene used in the test was cloned from *E. amylovora* based on published sequence[7] by PCR. A promoter fragment (ca. 450-bp length) of *prp*1-1 gene activated selectively upon *P. infestans* infection was cloned from potato cultivar "Desiree" using two oligonucleotides primers (5'-GCAAGCTTGATCCAAAATCTAACAAT-3' and 5'-CATCTAGAGTCTCCCTTGGTGTCT AGTCTT-3') designed by the published sequence[13]. The promoter was used to construct pathogen infection-induced harpin gene expression vector. Because harpin protein induced hypersensitive response in intercellular space in leaves, we inferred that the action site for harpin protein might be located on cell membrane or cell wall. In order to make expressed harpin protein secreted into intercellular space, a signal peptide sequence of 90 bp from tobacco *pr*1-*a* gene was fused with harpin protein gene at 5' end[14]. The constitutive expression vector of harpin protein gene was also constructed using pBI525 where CaMV double 35S promoters drive the gene expression. Fig. 1 is the sketch map of three plant expression vectors of harpin protein gene.

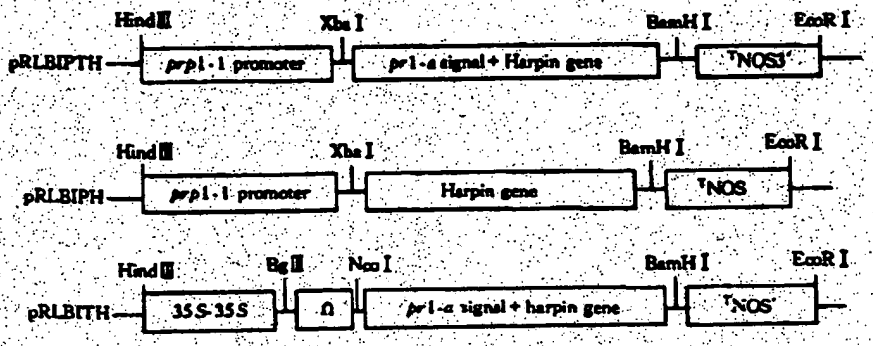

Fig. 1. Sketch map of harpin protein gene expression vectors.

Three recombinant pBINPLUS vectors (pBINPTH, pBINPH and pBINTH) were constructed by inserting gene expression cassette from pRLBIPTH, pRLBIPH, and pRLBITH respectively. The Ti plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 through freeze-thaw method.

Potato diploid line Line V and R2 were used as transformation materials. Stems with length of 0.5 cm were infected by *A. tumefaciens* LBA4404 containing expression vector. A total of 68 transgenic plants were produced through NPTII resistance selection. Five of those were regener-

ated from R2 genotype.

2.2 Detection of harpin protein gene expression in transgenic plants

mRNA transcription of harpin protein gene in transgenic plants was assayed by Northern hybridization. Harpin protein gene was transcribed into mRNA in 15 tested transgenic plants transformed with pBINTH (fig. 2(a)). Detection of harpin protein gene transcription in 11 transgenic plants transformed with pBINPTH and pBINPH showed that harpin gene mRNA was not detected before infection of *P. infestans* (fig. 2(b)), but it was detected in 48 h after infection. The result indicated that *prp*1-1 promoter was functional only after *P. infestans* infection in transgenic plants (figure 2(c)).

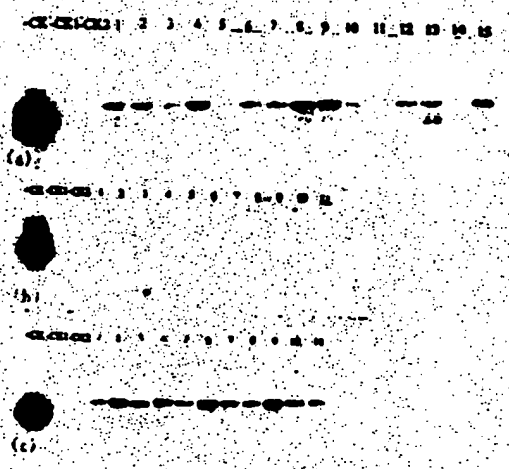

Fig. 2. Northern analysis of harpin gene transcription. (a) Transgenic plants transformed with pBINTH; (b) transgenic plants transformed with pBINPTH or pBINPH (before infection); (c) transgenic plants transformed with pBINPTH or pBINPH (48 h after infection).

Southern hybridization was also used to analyze the integration of harpin protein gene and demonstrated that at least 1—4 insertion events happened in transgenic genome (figure 3).

Dot blot-ELISA was employed to detect the harpin gene expression at protein level. Detection of 8 plants transformed with pBINTH demonstrated the accumulation of harpin protein in transgenic plants (fig. 4(a)). SDS-PAGE and Western blot analysis of total protein, intercellular protein and intracellular proteins revealed that harpin protein was secreted into intercellular space in transgenic plants (fig. 4(b)). Dot blot-ELISA detection of total protein extracted from 8 transgenic plants transformed with pBINPTH and pBINPH before and after infection of *P. infestans* showed that harpin protein in those transgenic plants was accumulated only after infection of *P. infestans* (figure 4(c)).

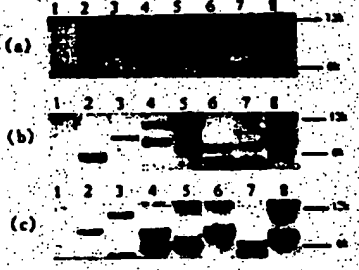

Fig. 3. Detection of integration of harpin gene in transgenic plants. 1, Untransformed plant; 2—8, transgenic plants. (a)—(c) pBINPTH, BINPH and pBINTH transformed plants, respectively.

2.3 Identification of disease resistance in transgenic plants

Late blight disease resistance was analyzed based on LGR value measured after infection of *P. infestans*. The average of LRG value across inoculated 15 compound leaves from negative control plant was 3.44 ± 0.44 for Line V and (4.16 ± 0.33) mm for R2. Based on statistical analysis for LGR, among 10 tested transgenic plants (Line V genotype) transformed with pBINTH, 6 plants (LRG = 1.31 − 2.55) showed significant resistance (difference at $\alpha = 0.05$), while 5 plants had extreme resistance (difference at $\alpha = 0.01$). Among 45 plants transformed with pBINPTH and pBINPH, 27 plants (LRG = 0.80—2.55) showed significant resistance (difference at $\alpha = 0.05$), while 25 plants had extreme resistance (difference at $\alpha = 0.01$). Two plants transformed with pBINPTH and pBINPH respec-

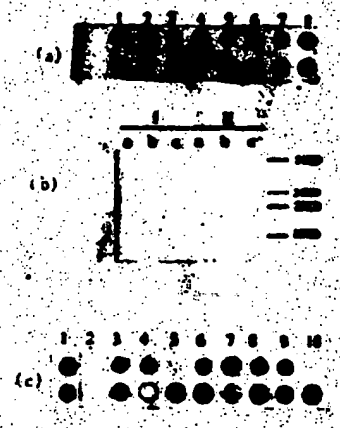

Fig. 4. Harpin protein accumulation and secretion in transgenic plants. (a) Dot blot-ELISA detection of harpin protein in transgenic plants; (b) Western blot analysis of harpin protein secretion in transgenic plants; I. untransformed plants; II. transgenic resistant plants; a. intercellular protein; b. intracellular protein; c. total protein. (c) Dot blot-ELISA detection of harpin protein accumulation in pBINPTH and pBINPH transformed plants before and after infection. 1. Positive control; 2. negative control; 3—10. prior to *P. infestans* infection (up row) and post *P. infestans* infection (bottom row).

tively were shown to be immune to infection of *P. infestans*. No hyphae was observed on inoculated leaves and necrotic lesion was limited to inoculation site.

3 Discussion

Using harpin protein gene, we explored a new approach of engineering hypersensitive response to develop resistant genotype to potato late blight disease. Statistical comparison of lesion growth rate between control plants and 55 transgenic plants indicated that there were 33 plants with significant resistance (difference at $\alpha = 0.05$), and 30 plants had extreme resistance (difference at $\alpha = 0.01$). The resistance in 19 plants (LGR<2.0) reached and even preceded that in "Robijin", a cultivar with good field resistance to *P. infestans*. Two plants transformed with pBINPTH and pBINPH respectively were shown to be immune to infection of *P. infestans*. The results provided evidence to indicate that pathogen infection-induced expression of harpin protein gene in transgenic plants inhibited the development of *P. infestans*. The research opened a new way towards development of disease resistant genotype.

We found in our experiment that constitutive expression of harpin protein in transgenic plants did not kill the plants or interfere with regeneration process of potato. The reason for that was probably due to the insufficient expression of harpin protein in those transgenic plants. We inferred that a critical concentration for harpin protein was necessary to induce hypersensitive cell death. Under the critical concentration, harpin could only induce disease resistance, but not cell death[15].

In order to locate the action site of harpin protein in cell, two transformation vectors were designed. One construct made expressed protein secrete into intercellular space, while another made it remain in cell. However, resistant transgenic plants were obtained from both cases and no evident difference in resistance between them was observed. Harpin protein might be involved in disease resistance signal transduction, but the mechanism is not clear.

Four amino acid difference between our harpin gene and that published[7] was due to different *E. amylovora* strains used. However, harpin protein mediated resistance in transgenic plants highlighted that the changes of 4 amino acids did not affect its function of inducing resistance.

Harpin protein-mediated resistance was reached through activation of defensive system of potato. In order to fully utilize the harpin protein mediated resistance, it is necessary to detect the physiological changes in transgenic plants such as PR protein expression, H^+/K^+ exchanges across cell membrane, accumulation of SA and so on. Particularly, it is important to detect the expression of PR1 and Osmotin protein in transgenic plants because some evidence indicated that both proteins were involved in resistance to *P. infestans* infection. An attention about resistance

in those transgenic plants should be paid to other potato diseases or other crop diseases since the harpin protein-mediated resistance may probably not be limited to late blight.

Acknowledgement The authors are grateful to Dr. Andy Pereira and Dr. Vivianne Vleeshouwers for their great help in potato transformation and disease resistance test. They also thank associate Prof. Wu Xingfeng for her kind help in preparation of this manuscript.

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My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HYPERSENSITIVE RESPONSE ELICITOR FROM *XANTHOMONAS CAMPESTRIS*

the specification of which (check only one item below):

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[X] was filed as U.S. Patent Application Serial No. **09/829,124** on **April 9, 2001** and was amended on _____ (if applicable).

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